Supplementary material

Methods

Healthy individuals, greater than 18 years of age, with a known melanoma were recruited from Washington University clinics. Each patient was evaluated by the principal investigator, Dr. Cornelius, prior to study involvement. Photoacoustic tomography (PAT) was performed prior to the scheduled excision of their melanoma. The excision was not delayed in order to perform PAT imaging of the melanoma. Prior to participation, patients were consented via the IRB approved consent forms (IRB ID#: 201410125). Ten patients were recruited and a total of thirteen lesions were imaged. Benefits and risks of the study were explained to the patients in detail and written consent was obtained. We performed PAT imaging of the melanomas using our handheld probe followed by an excision to determine the BD of each melanoma. Of the thirteen lesions that were imaged, six cases presented after an initial incisional biopsy, providing a provisional BD (pBD) prior to PAT imaging (see Supplementary Tables S1 & S2 online). Following imaging and histologic examination, we determined that one lesion was comprised entirely of melanophages with no tumor cells, and another amelanotic tumor extended to a depth (>10 mm) that was beyond the maximum imaging capacity of the PAT probe, although the shallower part of the melanoma can be still imaged. A third patient who presented with widely metastatic disease received systemic therapy at an outside institution prior to wide local excision of his primary lesion. Thus, we excluded these three cases in our final data analysis. An overview of the ten cases in the tables below includes five melanomas without the partial biopsies depths and five melanomas with partial biopsy depths.

To image the melanin-containing lesion, a handheld device was used (total skin contact surface area 1.25” x 0.5”). The determination of tumor depth and width by PAT at 680 nm wavelength depends upon the presence of melanin in the tumor, where blood has the lowest absorption. From the image, we were able to determine tumor depth or thickness (PA depth) as well as construct a 3D rendering of the tumor. These images along the z-x plane were then compared to the histological section at the same location (marked at the time of excision). The PAT measurement was adjusted by a factor of 0.88 to account for ex-vivo tissue fixation and processing (Winsor, 1994) and this is indicated as corrected (cPA) depth.

To increase imaging depth and detection reliability for highly pigmented lesions, skin was illuminated outside the pigmented part with high laser energy. Precaution was taken to guarantee that laser fluence at skin surface did not exceed American National Standards Institute (ANSI) safety limits. The optical fluence on the skin surface is approximately 10 mJ/cm^2, which is less than the safety limit set by the ANSI (20 mJ/cm^2) at this wavelength.

Maximum laser delivered pulse energy at full laser power was below 80 mJ at 650 nm, and light uniformly illuminates the skin area 2 cm x 2 cm which is within ANSI safety limits for skin exposure to laser light. Light delivery system was shielded to avoid eye exposure. Operator and patient used approved safety glasses with OD 6 at laser wavelengths. A DAQ module, which is in contact with patient, was installed in a personal computer and powered from it. Appropriate eye protection will be worn by patients and technicians at all times.

After the patient was deemed appropriate for imaging (L.A.C.), the patient presented for imaging, and at least two researchers consistently evaluated each patient (Y.Z and S.V.T.) to determine the deepest portion with *in vivo* measurement by PAT. These measurements were stored in a secure database. Surgical excision was then performed normally, as what is considered the standard of care. A dermatopathologist, (I.R.) blinded to the PAT measurement, provided the Breslow's depth after excision. These measurements were then compared to evaluate the accuracy of the PAT depth to the Breslow's depth and if provided initially, the partial biopsy depth (see Supplementary Tables S1 & S2 online).

Statistical analysis

Two important parameters are calculated based on the collected data, mean absolute error (MAE) and the coefficient of determination (R^2). For all the ten cutaneous melanoma metastasis PAT depths (cPA), the MAE is calculated as

$$MAE = \frac{1}{10}\sum_{i=1}^{10} | f_i - y_i | , \qquad \text{Eq. (1)}$$

where i is the patient index, f_i is the cPA from ith patient, and y_i is the excisional biopsy depth from ith patient. In total, we have data from 10 patients used for calculation.

The formula used for R^2 calculation is as follows:

$$R^2 = 1 - \frac{\sum_{i=1}^{10}(f_i - y_i)^2}{\sum_{i=1}^{10}(f_i - \bar{f})^2}, \qquad \text{Eq. (2)}$$

where $\bar{f}$ is the averaged value of cPA from all the ten measurements. For the five cases used for comparing cPA and incisional biopsy, the calculation for the MAE is similar just with different number of cases. The smaller MAE from PAT measurements than from the incisional biopsy indicates that PAT is more accurate in our study.

We also calculated the test confidence level in comparing the accuracy between PAT and incisional biopsy measurements. Our case can be treated as a two-sample t test, in which the test value can be calculated as:

$$t(2n-2) = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{(\frac{2}{n})\frac{\sum X_1^2 - (\sum X_1)^2/n + \sum X_2^2 - (\sum X_2)^2/n}{2n-2}}}, \qquad \text{Eq. (3)}$$

where n is the number of samples in each group, X_1 is the absolute difference between PAT measurement and excisional biopsy, X_2 is the absolute difference between incisional and excisional biopsy, $\overline{X}_1$ is the mean value for all X_1, and $\overline{X}_2$ is the mean value for all X_2. Substituting out data (n = 5, $\overline{X}_1$ = 0.14, $\overline{X}_2$ = 0.21, $\sum X_1^2$ = 0.1001, $(\sum X_1)^2$ = 0.4409, $\sum X_2^2$ = 0.2046, $(\sum X_2)^2$ = 1.2544), we have t(8) = -1.3723. For one-sided test, our result is close to student's t distribution value (-1.397) with 8 degrees of freedom and significant level of 0.1. Although our sample size is small, we still achieved relative good confidence level. We will perform more experiments to increase our confidence level in the future.

Photoacoustic tomography

Photoacoustic tomography (PAT) is a two-dimensional or three-dimensional imaging modality based on optical excitation and acoustic detection. The discovery of its underline principle, photoacoustic effect, can be traced back to 1880 by Alexander Bell. In PAT, usually a short pulsed laser illuminates the target. Following the absorption of the laser pulse, an initial temperature rise will generate an initial pressure rise based on the photoacoustic effect. The pressure rise will then propagate as an ultrasonic wave and finally get detected by a single ultrasonic transducer or a transducer array. On one hand, because PAT is based on optical absorption contrast, it has detected a variety of absorbers, including melanin, which is the main component of melanoma. On the other hand, because PAT is based on acoustic detection, it can image deep biological structures with high spatial resolution. Thus, PAT has successfully imaged melanoma in mice *in vivo* with thickness more than 7 mm.

Based on different imaging formation mechanism, PAT can be divided into point-scanning-based photoacoustic microscopy and reconstruction-based photoacoustic computed tomography

(PACT). In our melanoma study, we used a PACT system based on a linear-array (one-dimensional) probe. The basic parameters of this probe are introduced in the main text. The elevational direction is defined as the direction perpendicular to the probe, the lateral direction is the direction along the probe, and the axial direction is along the depth direction. The resolution definitions are different along different directions. The elevational and lateral resolutions, decided by the numerical aperture (NA) of the probe as well as the center acoustic wavelength (λ) of the probe, can be estimated by $0.72\lambda/NA$. The axial resolution, dependent on the frequency bandwidth (Δf) of the probe and the speed of sound (c) in soft tissue, is given by $0.88c/\Delta f$. Based on the probe parameters described in the manuscript, the resolutions can be calculated.

Age	Race	Gender	Melanoma Location	PA measurement cPA_{depth}	Final Pathology BD= Breslow's depth
59	White	Male	Left leg (lateral)	1.52 mm	1.67 mm
59	White	Male	Left leg (medial)	1.20 mm	1.73 mm
56	White	Female	Left calf	5.50 mm	5.00 mm
70	White	Male	Right leg (medial)	1.00 mm	0.96 mm
70	White	Male	Right leg (lateral)	1.20 mm	1.28 mm

Supplementary Table S1 online: Photoacoustic imaging on non-partially biopsied melanomas. cPA measurement is the corrected Photoacoustic measurement of the melanoma; BD is the final Breslow's depth after excision of the entire melanoma.

Age	Race	Gender	Melanoma Location	Partial biopsy (pBD)	PA measurement cPA_{depth}	Final Pathology BD= Breslow's depth
78	White	Male	Left temple	0.55 mm	0.40 mm	0.30 mm
36	White	Female	Left ankle	0.51 mm	0.80 mm	0.80 mm
97	White	Female	Left calf	0.24 mm	0.32 mm	0.35 mm
53	White	Female	Right foot	0.48 mm	0.56 mm	0.78 mm
53	White	Female	Left arm	0.30 mm	0.53 mm	0.18 mm

Supplementary Table S2 online: Photoacoustic imaging on partially biopsied primary melanomas. pBD = depth of partial biopsy; cPA measurement is the corrected Photoacoustic measurement of the melanoma; BD is the final Breslow's depth after excision of the entire melanoma.